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Deficiency of CD34^{+}*c-kit*$^{+}$ and CD34^{+}38^{-} Hematopoietic Precursors in Aplastic Anemia After Immunosuppressive Treatment

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To characterize the persistent abnormalities of hematopoiesis in aplastic anemia (AA) after immunosuppression with antilymphocyte globulin (ALG), we analyzed the quantity, phenotype, and growth properties of hematopoietic progenitor cells in 13 patients who received ALG treatment. Flow cytometry (FACS) revealed a deficiency of CD34^{+} cells in bone marrow (BM) of all patients. This deficiency was most severe (40-fold) in 4 patients in AA relapse. In 9 patients in remission, CD34^{+} cells were reduced 2–10-fold and showed no correlation with the ALG-induced improvement of peripheral blood cell counts. The proportion of CD34^{+} cells carrying *c-kit* receptors was abnormally low (2–10-fold below normal) in 5 of 13 AA patients. These patients also displayed low levels of *c-kit* mRNA by reverse transcription-polymerase chain reaction (RT-PCR). Furthermore, the CD34^{+} cell population was almost completely depleted of CD34^{+}CD38^{-} early hematopoietic progenitors in all AA patients. The proportion of CD34^{+} cells expressing lineage differentiation antigens CD33, CD71, and CD45RA in AA was increased, as compared to control BM. Formation of hematopoietic colonies by FACS-purified CD34^{+} cells was nearly absent in 4 relapsed patients, normal in 4 of 9, and decreased (up to 10-fold) in 5 of 9 patients in remission. The degree of impairment of colony-forming ability by AA progenitors correlated well with the reduction of CD34^{+}*c-kit*$^{+}$ cells. The best proliferative response of CD34^{+} cells was observed in the presence of stem cell factor and, in some cases, flt3 ligand. Our results indicate that the disease process in AA depletes immature BM progenitors, thus providing a plausible explanation for persistent defects in colony-forming ability and long-term regenerative capacity of AA marrow after immunosuppression. Analysis of the immunophenotypes and the proliferative properties of purified progenitors may be useful for estimating degree of hematopoietic recovery in ALG-treated patients.

Key words: aplastic anemia, immunosuppression, CD34^{+} cells, *c-kit*, stem cell factor, flt3 ligand

INTRODUCTION

Multilineage bone marrow (BM) failure in aplastic anemia (AA) and the inability of residual marrow cells to grow in vitro indicate that the disease affects early stages of hematopoiesis [1]. BM function can be improved by immunosuppressive therapy, as judged by near-normal peripheral blood counts and marrow cellularity in remission. However, hematopoiesis after treatment with ALG and cyclosporin A remains fragile. Patients continue to show signs of residual disease activity: clinically, they are at high risk of relapse and of late clonal disorders [2–4], and in vitro, the proliferative capacity of BM cells remains low [5,6]. Immune-mediated suppression of BM function by autoaggressive T cells is considered one of the most important pathogenetic mechanisms of AA [7,8]. Processes underlying the persistent hematopoietic lesion after immunosuppressive treatment are poorly understood. Possible reasons for a slow and incomplete recovery may include an unidentified treatment-resistant defect

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of hematopoietic stem cells, or their irreversible loss caused by an autoimmune process at onset of the disease. Limited availability of AA marrow hampers analysis of early hematopoietic progenitors; most studies have been performed using total unfractionated BM cells in clonogenic and long-term culture assays [9,10]. The stem-cell compartment in AA has only recently begun to be characterized by immunophenotypic and functional analysis of isolated precursors. It has been shown that the population of CD34$^+$ hematopoietic cells is reduced [11,12], is deficient in colony-forming cells [11], and fails to survive on preformed stroma layers [13], thus suggesting quantitative and functional abnormalities of CD34$^+$ cells in AA.

The CD34 antigen is expressed on approximately 1–4% of human BM cells and identifies a heterogenous population of multipotent hematopoietic progenitors capable of forming colonies in vitro and reconstituting hematopoiesis in vivo [14,15]. Phenotypic changes during the stepwise differentiation of stem cells are characterized by loss of some and acquisition of other specific cell-surface antigens. The most immature CD34$^+$ cells express no lineage-specific antigens. They acquire CD38 antigen at an early step of differentiation [16], and later express the lineage-associated antigens CD45RA, CD33, and CD71, according to commitment to lymphoid and myeloid/erythroid lineages [17]. In parallel, expression of cell-surface receptors for hematopoietic growth factors defines the differentiation stage of BM precursors. The CD34$^+$ lineage-negative population expresses *c-kit,* the receptor for stem cell factor (SCF) [18,19]. *c-kit* transmits growth-enhancing signals and mediates adhesion of stem cells to stroma cells [20,21]. Its absence or its mutation in genetically-deficient *W* mice results in severe anemia [21], confirming the significance of *c-kit* receptors for early hematopoietic development. The *flk-2/flt-3* receptor and its recently identified flt3 ligand represent another receptor/ligand pair involved in regulation of the hematopoietic properties of CD34$^+$ cells [22,23].

In this study, we analyzed the CD34$^+$ cell compartment in BM of AA patients after immunosuppressive treatment with ALG. We characterized the frequency of CD34$^+$ cells and their subsets expressing the immature hematopoietic markers, CD38 and *c-kit,* and the markers of differentiation and lineage-commitment steps, CD33, CD71, and CD45RA. We isolated CD34$^+$ progenitors and examined their clonogenic properties in vitro in cultures supplemented with early-acting hematopoietic growth factors, including SCF and flt3 ligand. Our results indicate that CD34$^+$ cells of AA patients after ALG treatment are severely deficient in early hematopoietic precursors and, in most cases, remain poorly responsive or nonresponsive to hematopoietic stimuli, thus supporting clinical observations on incomplete hematologic recovery after immunosuppression.

MATERIALS AND METHODS

Patients and Controls

Thirteen patients with AA and 5 BM donors were studied after informed consent (see Table I). All had been treated with one or two courses of horse ALG (Bern, Switzerland) in combination with high doses of corticosteroids, according to a standard protocol [24]. At presentation, all patients had severe AA [25] and required red blood cell and platelet transfusions. At time of study, 9 patients were in remission and had become transfusion-independent as a response to ALG therapy. Since time of study, 1 patient (patient 1) has relapsed, and 2 patients (patients 10 and 11) died of AA-related complications. None of the patients showed signs of acquired clonal disorders by a negative Ham test, and by absence of cytogenetic abnormalities.

Bone Marrow Cells

BM was aspirated in not more than 5-ml aliquots, and mononuclear cells (BM-MNC) were isolated by Ficoll-Hypaque (d = 1.077; Pharmacia, Uppsala, Sweden) density gradient centrifugation. Cells were cryopreserved in Iscove's modified Dulbecco's medium (IMDM; GIBCO, Grand Island, NY) containing 40% fetal calf serum (FCS) and 20% dimethyl sulfoxide. Before each experiment, cells were rapidly thawed and slowly diluted in IMDM supplemented with 25% FCS and 0.01 mg/ml DNase I (type II; Sigma, St Louis, MO) to avoid cell aggregation. Viability of cells was >80% in the trypan blue exclusion assay.

Monoclonal Antibodies (MoAbs)

For immunophenotyping analysis of BM-MNC, the following fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mouse anti-human MoAbs were added at pretested concentrations: anti-CD34-PE or -FITC (8G12, HPCA-2, IgG1), anti-CD33-PE (anti-Leu-M9, IgG1), anti-CD45RA-FITC (anti-Leu-18, IgG1), anti-CD71-FITC (anti-transferrin receptor, IgG2a), and control mouse-IgG1-FITC or -PE, all purchased from Becton Dickinson (San Jose, CA); and anti-CD38-FITC (T16, IgG1) and anti-*c-kit*-PE (95C3, IgG1), both from Immunotech (Marseille, France). For cell-sorting experiments, anti-CD34-FITC (HPCA-2, Becton Dickinson) was used.

Cell Fluorescence (FACS) Analysis and Sorting

Flow cytometric cell analysis was performed with FACScan and cell-sorting with FACStar Plus (both Becton Dickinson), equipped with a 488-nm argon-ion laser. Before staining, BM-MNC were incubated for 15 min with heat-inactivated rabbit serum (5 μl/1 × 10^6 cells; GIBCO) to block fragment cristalline (Fc) receptors. Dual-color FACS analysis was performed using specific and control isotype-matched MoAbs, as indicated in the

TABLE I. Patient Characteristics*

Patient no. (UPN)	1 (356)	2 (328)	3 (145)	4 (499)	5 (291)	6 (136)	7 (162)	8 (109)	9 (−)	10 (48)	11 (331)	12 (258)	13 (468)
Diagnosis at presentation	SAA/i	SAA/i	SAA/i	SAA/i	SAA/i	SAA/d	SAA/i	SAA/i	SAA/i	SAA/i	SAA/i	SAA/i	SAA/i
Age (y) at presentation/sex	2/M	55/F	25/M	63/M	55/F	21/M	10/F	9/F	13/F	24/F	32/M	15/F	67/F
Treatment prior to study	ALG	ALG	ALG × 2	ALG	ALG	ALG	ALG	ALG × 2	ALG	ALG × 2	ALG × 2	ALG × 3	ALG
Hematological status at time of study													
Time since ALG treatment	1 y	6 y	8 y	3 m	5 y	7 y	6 y	11 y	14 m	11 y	14 m	10 y	3 y
Neutrophils (× 10^9/l)	6.1	4.1	6.0	27.1	3.5	3.4	4.5	4.4	15.0	1.0	0.2	1.4	0.3
Reticulocytes (× 10^9/l)	39	34	96	107	54	75	32	73	82	45	12	30	22
Platelets (× 10^9/l)	53	167	155	73	309	63	131	363	38	75	42	8	19
Hb (g %)	12.1	12.7	10.2	9.6	13.8	14.7	14.1	11.9	10.0	12.8	10.6	8.9	9.4
Transfusion requirement	No	No	No	No	No	No	No	No	No	Yes	Yes	Yes	Yes
Bone marrow cellularity	100%	100%	80%	>100%	60%	100%	100%	90%	>100%	40%	15%	50%	5%
Medication	CyA, A	None	CyA, A	CyA, G-CSF	None	None	None	None	CyA, G-CSF	CyA, A	CyA, A	G-CSF	CyA, A
Current clinical condition	R^P	R	R	R	R	R	R	R	R	Died	Died	R^P	Rel

*UPN, unique patient number; i, idiopathic; d, drug-induced; M, male; F, female; y, years; m, months; CyA, cyclosporin A; A, androgens; R, remission; R^P, partial remission; Rel, relapse.

figures and tables. For cell sorting, 1.0–1.5 × 10^7 BM-MNC were incubated with anti-CD34 (FITC) MoAb for 30 min at 4°C, washed twice, and resuspended at a 2 × 10^6/ml in phosphate-buffered saline (PBS) containing 5% FCS. Gates were set according to forward light scatter (FSC) and sideward light scatter (SSC) to exclude residual granulocytes, erythroblasts, and apoptotic and dead cells. For FACS analysis, 50,000 events were acquired in list mode data files using Lysis II software (Becton Dickinson, San Jose, CA). For FACS-sorting, in addition to gates established according to light-scattering properties of $CD34^+$ cells, sorting windows were set for FITC-positive fluorescent $CD34^+$ cells. During cell-sorting procedure, cells were maintained at 4°C (sorting rate, 1,000 cells/sec). Reanalysis of sorted cells with Consort software (Becton Dickinson, San Jose, CA) estimated an average purity of 91.4% and 85.5% of control and AA $CD34^+$ cells, respectively.

Recombinant Human Hematopoietic Growth Factors

The following factors and their final concentrations were used: SCF (AMGEN, Thousand Oaks, CA) at 50 ng/ml; interleukin-3 (IL-3) at 25 U/ml; granulocyte-macrophage colony-stimulating factor (GM-CSF) at 25 U/ml; interleukin-1β (IL-1β) at 100 ng/ml; interleukin-6 (IL-6) at 100 U/ml (all from Sandoz, Basel, Switzerland); granulocyte colony-stimulating factor (G-CSF; Chugai-Rhone-Poulenc Rorer, Antony, France) at 2.1 ng/ml; interleukin-11 (IL-11; Genetics Institute, Cambridge, MA) at 100 ng/ml; Flt 3 ligand (gift of D. Lyman, Immunex, Seattle, WA) at 60 ng/ml and erythropoietin (Epo; Connaught, Willowdale, Ontario, Canada) at 1.4 U/ml.

Hematopoietic Colony-Forming Assay

Unfractionated BM-MNC (1 × 10^5) or purified $CD34^+$ (1 × 10^3) cells were plated in 1.25 ml methylcellulose cultures as described [10,26]. Human recombinant hematopoietic growth factors (see above) or 10% of conditioned medium (CM) from human peripheral blood mononuclear cells, stimulated with 20% autologous serum and 1% phytohemagglutinin (PHA), were added. After 14 days in culture at 37°C with 5% CO_2, colonies of >50 cells were counted.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total cellular RNA isolated using RNAzol [27] (Paesel & Lorei, Frankfurt, Germany) from 5 × 10^5 BM-MNC was reverse-transcribed in 20 μl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 20 U RNAguard (Pharmacia, Uppsala, Sweden), 0.5 mM each of dATP, dGTP, and dTTP, 0.25 mM dCTP, 2.5 μM random hexamers (Perkin Elmer, Branchburg, NJ), and 50 U M-MLV reverse transcriptase (GIBCO, Gaithers-

burg, MD). Four μl and 16 μl of RT reaction were used for PCR-amplifications of β-actin and *c-kit* transcripts in a total volume of 20 μl and 80 μl, respectively. The following PCR primers were used: for β-actin, sense 5′-TTGAGACCTTCAACACCCCAGCC-3′ position 2036–2058, antisense 5′-CCCAGGAAGGAAGGCTGGAAGA-3′, position 2447–2468; for *c-kit* (amplification of the fragment corresponding to the kinase and part of the transmembrane region of the receptor [28], sense 5′-AGTACATGGACATGAAACCTGG-3′, pos. 2180–2201, antisense 5′-GATTCTGCTCAGACATCGTCG-3′, position 2940–2960. PCR reactions containing specific primers (0.3 μM each), AmpliTaq DNA polymerase (0.025 U/μl; Perkin Elmer), and ^{32}P-dCTP (0.15 μCi/μl; specific activity 3,000 Ci/mmol; Du Pont NEN, Boston, MA) were overlaid with Ampli Wax PCR gems (Perkin Elmer). Twenty-two PCR cycles were performed: denaturation at 94°C for 1 min, annealing at 60°C for 1 min and 30 sec, and extension at 72°C for 1 min and 30 sec. Half of the β-actin PCR product (10 μl) and all of the ethanol-precipitated *c-kit* PCR product were separated by 5% nondenaturing polyacrylamide gel electrophoresis (PAGE). Gels were dried and exposed onto a Phosphor Imager Screen (Bio-Rad, Hercules, CA) for 3 hr. The relative ratio of *c-kit* vs. β-actin signals was determined in pixel density units (PDUnits) with Phosphor Analyst software (Bio-Rad, Hercules, CA). Autoradiography was performed for 4 hr at −70°C, using Kodak X-ray film and amplifying screens (Sigma, St. Louis, MO).

Statistical Analysis

Results are expressed as the mean ± SEM of data obtained from multiple, separate experiments. Statistical significance was determined using the unpaired Student's t-test.

RESULTS

Frequency of CD34$^+$ and CD34$^+$*c-kit*$^+$ Cells and Its Correlation With the Proliferative Capacity of BM Cells

We examined BM of 13 AA patients after ALG treatment. Nine of them had achieved transfusion-independent hematopoietic function, accompanied by mild thrombocytopenia in 4 patients. Four patients required transfusion at time of investigation, and 2 of them (patients 10 and 11) later died of AA-related complications (Table I).

Hematopoietic progenitors were quantitated by flow cytometry of BM-MNC immunostained with monoclonal antibodies against CD34 and *c-kit* cell-surface proteins (Fig. 1). In parallel, BM samples were examined for formation of hematopoietic colonies in methylcellulose cultures. Results obtained with 13 AA and 5 control BM are presented in Table II, in which patients are arranged according to decreasing content of CD34$^+$ cells. The frequency of CD34$^+$ progenitors in BM of AA patients was 7.3-fold lower than normal (0.77 ± 0.18% vs. 5.62 ± 0.88%). The content of CD34$^+$ cells was reduced 40-fold in patients 10–13, who continued to have severe, transfusion-dependent aplasia. Hematologic recovery in patients 1–9 was associated with higher, but not fully normalized, CD34$^+$ cell numbers, which varied widely from patient to patient. We found no correlation between frequency of CD34$^+$ progenitors and peripheral blood values during ALG-induced remission.

Colony formation by BM-MNC cultured in the presence of PHA-induced conditioned medium was, on average, 10.8-fold lower than normal (23 ± 8 vs. 249 ± 47 colonies/10^5 cells; Table II), and the impairment of colony-forming ability could not be overcome by addition of a combination of recombinant growth factors, including SCF, Epo, IL-3, and G- and GM-CSF. Plating efficiency of BM cells was very low in patients 10–13, with severe deficiency of CD34$^+$ cells. Colony-forming ability correlated with content of CD34$^+$ cells ($R = 0.91$), but in several patients in remission (patients 3, 4, 6, and 9), colony numbers were lower than expected from the percentage of CD34$^+$ progenitors.

The frequency of CD34$^+$*c-kit*$^+$ cells in AA was 8-fold lower than normal (0.36 ± 0.13% vs. 2.88 ± 0.55%; Table II). On average, percentage of CD34$^+$*c-kit*$^+$ cells within the CD34$^+$ population was similar in patients and controls, although individual values in several patients were abnormally low; the deficiency of CD34$^+$*c-kit*$^+$ content was particularly pronounced in poorly-growing BM-MNC of patients 3, 4, 6, 9, and 11. BM of these patients contained a 2–10-fold lower proportion of *c-kit* receptor-carrying cells within their CD34$^+$ population, as compared with controls. The percentage of CD34$^+$*c-kit*$^+$ cells correlates well with the colony-forming capacity of AA marrow ($R = 0.96$). Deficiency of *c-kit*$^+$ cells in AA was confirmed by low *c-kit* mRNA expression levels, as analyzed by RT-PCR. PCR conditions were established to allow a linear relationship between amount of amplification product and number of PCR cycles (Fig. 2A). Expression of *c-kit* and, as a reference, β-actin mRNA was quantitated by measuring incorporation of ^{32}P-CTP into specific PCR fragments. *c-kit*/β-actin mRNA ratios in AA were 0.3–3.3% (mean, 0.89%) vs. 2.1–3.78% (mean, 2.74%) in controls (see legend, Fig. 2B). The lowest levels of *c-kit* mRNA were observed in BM from patients 3, 4, 6, 9, and 10, containing the lowest percentage of CD34$^+$*c-kit*$^+$ cells.

Proliferative Properties of Purified CD34$^+$ Cells

To normalize for differences in frequency of CD34$^+$ cells in BM from individual AA patients, we purified CD34$^+$ cells by FACS-sorting and analyzed their colony-forming properties after plating the same amount of AA and control cells. Purification of CD34$^+$ cells also avoided interference by lymphocytes and other accessory cells with potential growth-inhibiting properties. Methylcellu-

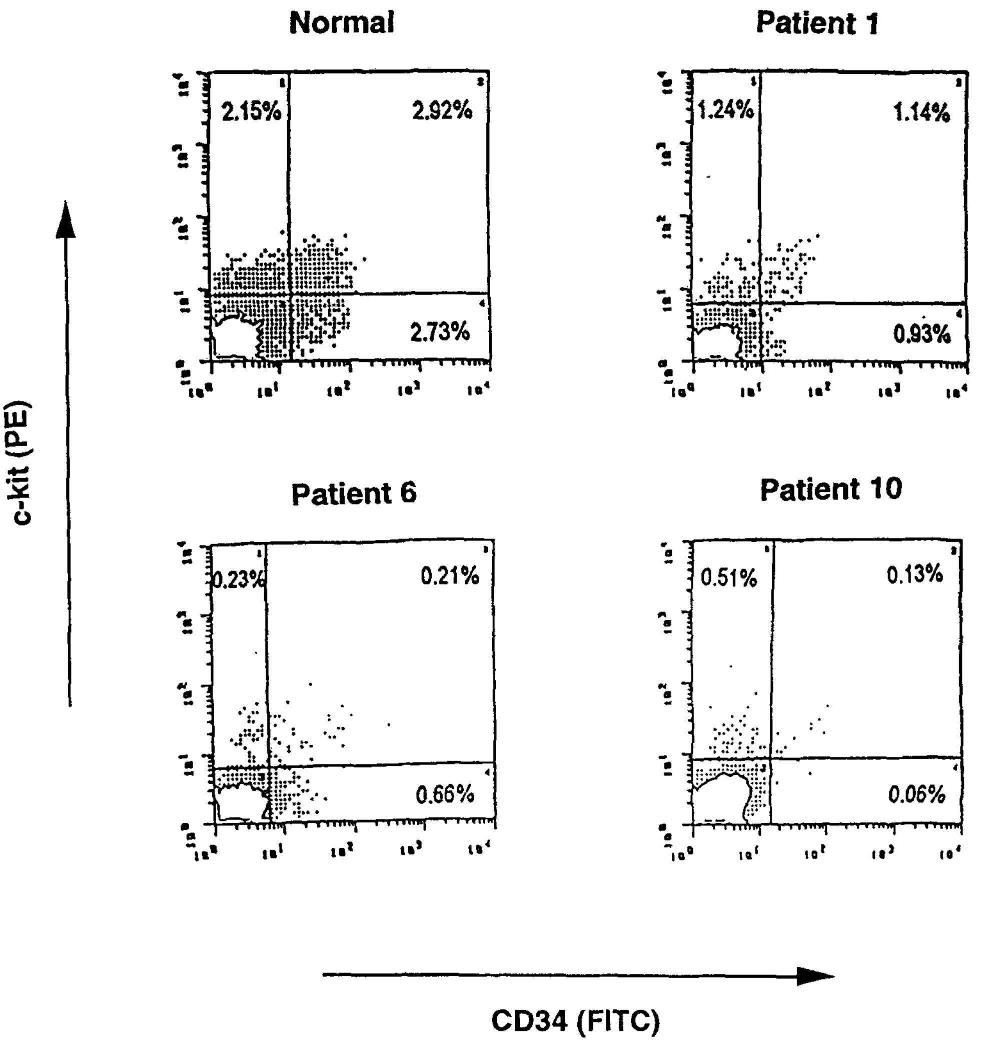

Fig. 1. Flow-cytometric analysis of CD34⁺ and CD34⁺*c-kit*⁺ cells. BM-MNC from normal and AA patients marrow were double-stained with anti-CD34 (FITC) and anti-*c-kit* (PE) antibodies. Numbers of quadrants refer to percentage of each of the single- and double-stained cell populations.

lose cultures were supplemented with early- and late-acting hematopoietic growth factors used in various combinations (Table III). On average, the plating efficiency of AA cells was only 2–3-fold lower than normal (as compared with an over 9-fold difference found using total BM-MNC). The highest colony numbers were obtained with factor combinations containing SCF. Flt3 ligand had an enhancing effect on colony growth when used together with interleukin-1, -3, -6, and -11 and G- or GM-CSF, but was always less effective than SCF. There was a high patient-to-patient variability of growth response of purified CD34⁺ cells (Fig. 3). In the presence of the most efficient stimulatory growth factor combinations, colony formation reached normal or near-normal values in 4 patients. Plating efficiency remained reduced up to 10-fold in the other 5 patients in remission, including patients 3, 4, 6, and 9, along with the abnormally low frequency of *c-kit*⁺ cells within the CD34⁺ population (Table II). Only single colonies were formed by purified CD34⁺ cells from the 2 patients with relapse of AA. Growth differences were confirmed by ^{3}H-thymidine uptake in suspension cultures; growth from CD34⁺ cells of patient 1 was delayed but reached normal levels, while proliferation of cells from patient 10 was 2% of normal (results not shown).

We investigated whether precursor cells of different commitments are equally affected by the disease process of AA, by morphologically characterizing types of hematopoietic colonies growing from purified CD34⁺ cells. As shown in Figure 4, there was no difference in relative frequency of colonies formed by CFU-GM, BFU-E, and CFU-GEMM precursors from normal and AA cells, irrespective of their total colony-forming ability. This indicates that even poorly-growing residual CD34⁺ cells in BM of AA patients maintain multipotential hematopoietic capacity.

TABLE II. Frequency of CD34$^+$ and CD34$^+$*c-kit*$^+$ Cells and Colony-Forming Ability of BM in AA*

			Colonies/10^5 BM-MNC	
Bone marrow	CD34$^+$ (%)	CD34$^+$*c-kit*$^+$ (%)	CM	Epo, SCF, IL-3, G-, GM-CSF
AA patient				
1	2.07	1.14	83	n.d.
2	2.05	1.49	73	202
3	1.02	0.08	11	n.d.
4	0.91	0.23	22	44
5	0.87	0.57	38	109
6	0.87	0.21	15	n.d.
7	0.64	0.26	18	83
8	0.58	0.38	27	70
9	0.49	0.10	2	6
10	0.19	0.13	7	19
11	0.17	0.01	0	2
12	0.08	0.04	0	1
13	0.01	0.01	1	4
Mean ± SEM	0.77 ± 0.18[a]	0.36 ± 0.13[a]	23 ± 8[a]	54 ± 20[a]
N, Range	(3.15–8.64)	(1.01 ± 4.22)	(89–348)	(369–495)
Mean ± SEM	5.62 ± 0.88[a]	2.88 ± 0.55[a]	249 ± 47[a]	420 ± 39[a]

*Normal (N; n = 5) and AA (patients 1–13) BM-MNC were analyzed for percentage of CD34$^+$ and CD34$^+$ *c-kit*$^+$ cells using flow cytometry, and for colony formation in methylcellulose cultures supplemented with PHA-induced conditioned medium (CM) or a mixture of hematopoietic growth factors (see Materials and Methods). n.d., not done.

[a]Statistical significance of all analyzed parameters in AA vs. N, $P < 0.0001$.

Expression of Hematopoietic Differentiation Antigens

To assess the differentiation stage of AA precursor cells, we used flow cytometry to determine the frequency of CD34$^+$ cells coexpressing early differentiation marker CD38 and the lymphoid and myeloid/erythroid lineage-specific markers, CD33, CD71, and CD45RA. There was a pronounced deficiency of the very immature population of CD34$^+$38$^-$ hematopoietic progenitors (Fig. 5). The frequency of CD34$^+$38$^-$ cells in BM-MNC was 38-fold below normal levels (0.05 ± 0.02% vs. 1.90 ± 0.49%). The content of CD34$^+$CD38$^-$ cells within the CD34$^+$ population was 7-fold lower than in controls (4.3 ± 1.7% vs. 30.8 ± 9.5%). Deficiency of CD34$^+$CD38$^-$ progenitors was observed in BM of all analyzed AA patients, irrespective of their hematological status and frequency of CD34$^+$ cells (Fig. 6).

With respect to CD33, CD71, and CD45RA antigenic determinants, the level of CD34$^+$ cell subsets within BM-MNC was 4–9-fold lower than in controls (not shown), as could be expected from the reduced frequency of the CD34$^+$ population in AA. Within CD34$^+$ cells, the proportion of lineage-negative subsets was decreased, and lineage-positive subsets were increased in AA as compared to controls (Table IV). The differences were not higher than 2-fold, but were consistently observed in every analyzed cell population.

DISCUSSION

Immunosuppression with ALG is an established therapy for patients with severe AA who are not eligible for bone marrow transplantation (BMT). Whereas BMT is curative, the remission induced by ALG is incomplete and prone to clinical complications, and remains associated with low proliferative capacity of BM cells in vitro. In this study, we examined the frequency, immunophenotypic composition, and growth properties of CD34$^+$ cells in BM of patients with AA of different severities after ALG treatment. We found quantitative and functional abnormalities in hematopoietic progenitors of all patients, 9 of whom were in clinical remission, and 4 of whom were transfusion-dependent after ALG.

As might be expected, the content of CD34$^+$ cells was particularly low (40-fold below normal) in the 4 patients who had not recovered, or had relapsed with AA after ALG. They resembled the newly-diagnosed patients with severe AA with up to a 100-fold reduction in CD34$^+$ cells (in Maciejewski et al. [11] and our unpublished data). Not only the number, but also the clonogenic capacity, of the few remaining CD34$^+$ cells were almost absent in patients with relapse of AA. The quantity and the proliferative capacity of CD34$^+$ cells can improve after immunosuppressive therapy [11]. We also observed that the number and colony-forming capacity of CD34$^+$ cells in

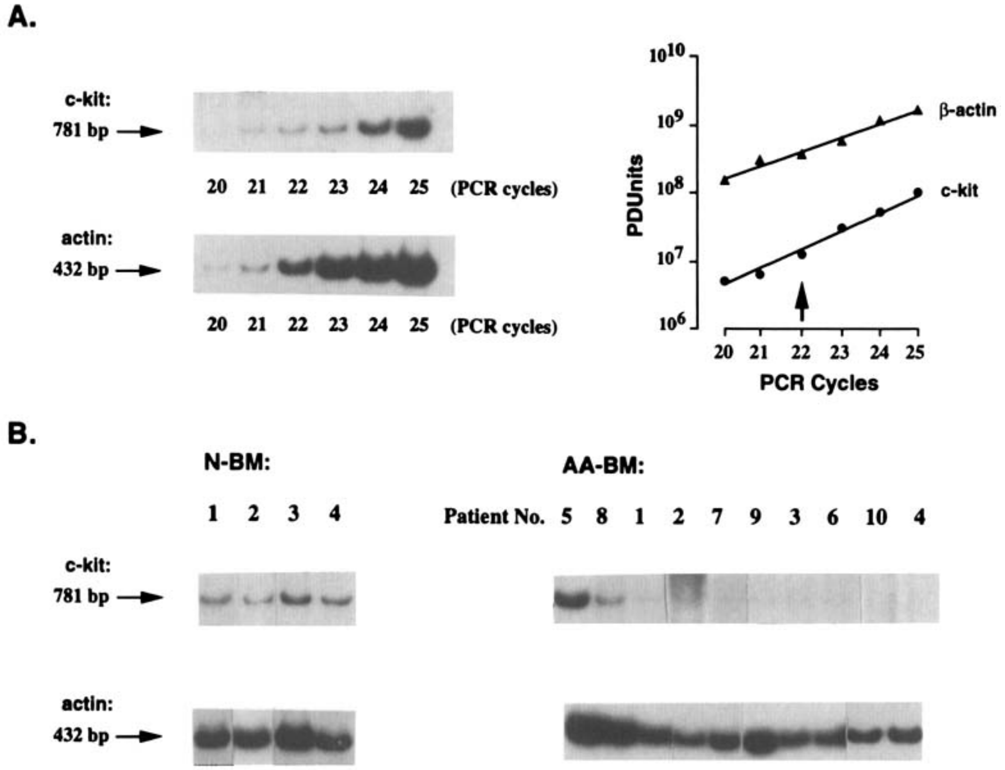

Fig. 2. RT-PCR analysis of *c-kit* mRNA expression. RNA from 5×10^5 BM-MNC of normal (N) and AA marrow was subjected to RT-PCR using ^{32}P-dCTP (see Materials and Methods). A: Analysis was performed with normal RNA, using increasing numbers of PCR cycles; radioactivity in each band was quantified in PDUnits by phosphorimaging analysis. Twenty-two cycles were chosen for further analysis as conditions of the linear accumulation of both *c-kit* and β-actin PCR products. B: Analysis was performed with 22 cycles using RNA from N and AA cells, as indicated. Arrows point to expected size fragments amplified from *c-kit* and β-actin mRNAs. *c-kit*/β-actin mRNA ratios were: N, 2.74% (range, 2.1–3.68%); AA patients: 1, 0.96%; 2, 0.46%; 3, 0.30%; 4, 0.52%; 5, 3.33%; 6, 0.29%; 7, 0.90%; 8, 1.51%; 9, 0.32%; and 10, 0.35%. We are unable to explain the strong *c-kit* mRNA signal reproducibly observed with BM-MNC of patient 5, which is higher than expected from the frequency of *c-kit*$^+$ cells.

TABLE III. Effect of Combinations of Hematopoietic Growth Factors on Colony Formation by CD34$^+$ Cells*

	N	AA
—	0.8 ± 0.8	0.7 ± 0.4
SCF	46.3 ± 9.0	17.0 ± 4.5
IL-3, G-, GM-CSF	92.8 ± 23.1	31.0 ± 11.2
IL-3, G-, GM-CSF, SCF	152.5 ± 22.6	61.3 ± 17.6
IL-1, IL-3, IL-6	48.3 ± 16.5	15.3 ± 5.3
IL-1, IL-3, IL-6, SCF	153.0 ± 35.4	50.1 ± 14.5
IL-1, IL-3, IL-6, flt3 ligand	105.3 ± 20.1	17.8 ± 6.6
IL-3, IL-11, GM-CSF	41.3 ± 11.9	14.1 ± 6.2
IL-3, IL-11, GM-CSF, SCF	117.3 ± 34.8	47.2 ± 16.0
IL-3, IL-11, GM-CSF, flt3 ligand	84.7 ± 28.5	32.8 ± 13.7

*Results are presented as mean ± SEM of total colony numbers formed by normal (N; n = 5) and AA (patients 1–10) CD34$^+$ cells. Epo was added to all growth factor combinations. For concentrations of growth factors see Materials and Methods.

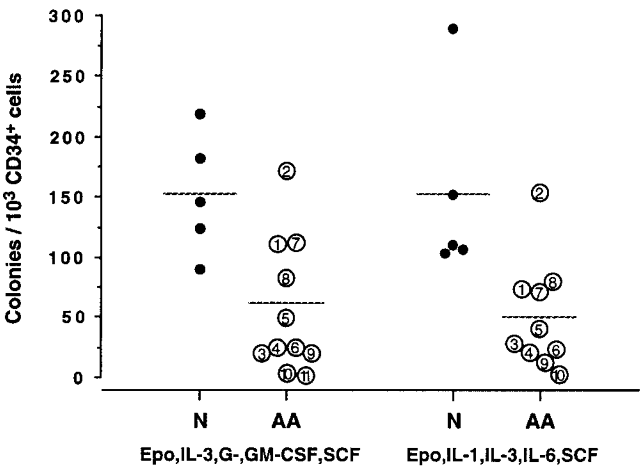

Fig. 3. Colony-forming ability of CD34$^+$ cells. Total colony numbers formed in response to indicated combinations of hematopoietic growth factors by FACS-purified CD34$^+$ cells from normal (N, black dots) and AA (patient numbers in open circles) bone marrow.

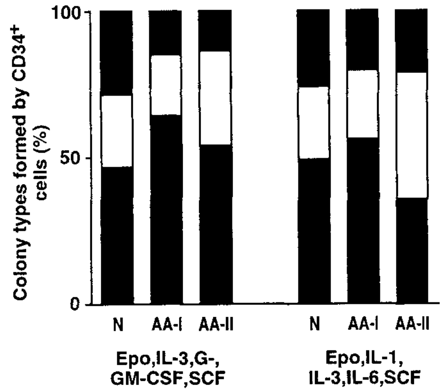

Fig. 4. Types of hematopoietic colonies formed by CD34⁺ cells. Results are presented as percentage of each colony type within total colonies formed by FACS-purified CD34⁺ cells in response to indicated combinations of growth factors. N, normal; AA-I, well-growing (patients 1, 2, 7, and 8) and AA-II, poorly-growing (patients 3–6 and 9) CD34⁺ cells. ■, GM-CFU; □, BFU-E; ▩, GEMM-CFU.

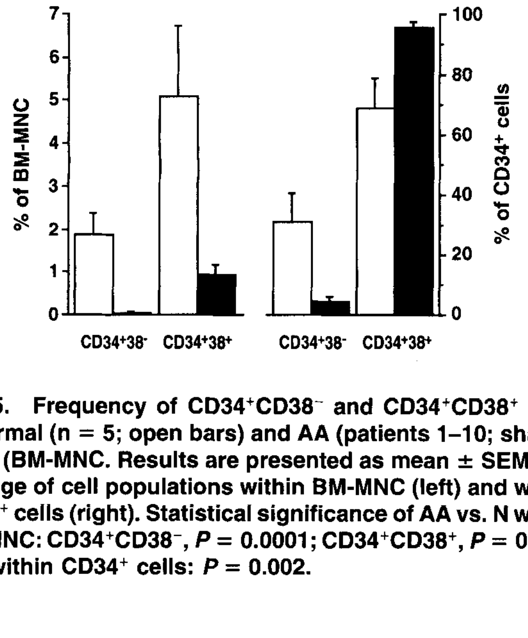

Fig. 5. Frequency of CD34⁺CD38⁻ and CD34⁺CD38⁺ cells in normal (n = 5; open bars) and AA (patients 1–10; shaded bars) (BM-MNC. Results are presented as mean ± SEM percentage of cell populations within BM-MNC (left) and within CD34⁺ cells (right). Statistical significance of AA vs. N within BM-MNC: CD34⁺CD38⁻, $P = 0.0001$; CD34⁺CD38⁺, $P = 0.037$; and within CD34⁺ cells: $P = 0.002$.

patients in clinical remission were higher than in patients in relapse. However, in most patients who have achieved near-normal hematopoietic function after ALG therapy, the frequency and plating efficiency of CD34⁺ cells remained reduced up to 10-fold for as long as 11 years post-treatment.

There was a high variability in colony-forming capacity of purified CD34⁺ cells from individual AA patients in remission. This variability correlated with the content of CD34⁺*c-kit*⁺ cell subset within the CD34⁺ population. Our results indicate that a persistent proliferative BM defect can be, at least in part, caused by a deficiency in CD34⁺*c-kit*⁺ cells, a population of hematopoietic progenitors which is highly enriched in colony-forming cells [29]. To answer the question of whether proliferative defects add to the deficiency of these cells in AA, one would have to test clonogenicity of isolated CD34⁺*c-kit*⁺ cells. Such experiments are difficult in view of the limited availability of progenitors in AA. Very poor growth of isolated CD34⁺ cells from 2 patients in relapse of AA (including 1 patient with a normal proportion of *c-kit*-receptor carrying cells) suggests that defects intrinsic to hematopoietic stem cells may contribute to the disease process, resulting in treatment-refractory clinical progression.

We confirmed the abnormally low frequency of CD34⁺*c-kit*⁺ cells in AA by analysis of *c-kit* expression at the mRNA level. There is, however, no evidence that BM failure in AA is associated with abnormalities at the *c-kit* gene locus, resembling defects responsible for anemia in *W* murine mutants. We performed single-strand conformation polymorphism (SSCP) analysis of *c-kit* cDNA in patients with severe AA, including patient 11 of this study. We did not detect any point mutations or deletions within exons encoding the transmembrane and kinase domains, which could potentially alter the signalling function of *c-kit* receptors (C. de Castro, R.E. Kaufman, and A. Wodnar-Filipowicz, unpublished results). These results indicate that deficiency of *c-kit* receptor-carrying CD34⁺ cells, rather than abnormalities at the *c-kit* gene expression level, may play a role in the pathophysiology of AA.

Analysis of the colony-forming ability of purified CD34⁺ cells, instead of unseparated BM, helps to detect minimal residual hematopoietic function in AA. In an attempt to improve the clonogenic capacity of residual hematopoietic progenitors in AA, we examined the effect of different combinations of hematopoietic growth factors chosen to obtain a maximum in vitro proliferation of early precursor cells. In agreement with our previous results [10], SCF was found to be the most efficient stimulator of CD34⁺ cells in vitro, promoting multilineage differentiation of even poorly-growing AA progenitors. In some patients, flt3 ligand was also found to enhance the effect of the cytokines IL-1, -3, -6, -11, G- and GM-CSF. Therefore, adjuvant use of SCF or flt3 ligand may be of value for patients who have recovered some BM function after therapy with ALG.

BM of all analyzed patients, irrespective of their hematological status and frequency of CD34⁺ population, was virtually devoid of precursors with the CD34⁺CD38⁻ phenotype. Depletion of CD34⁺CD38⁻ cells provides the strongest evidence so far for a persisting hematopoietic lesion common to all AA patients after ALG treatment. In chimeric patients treated with BMT for AA, frequency of CD34⁺CD38⁻ cells is normal (data not shown), indicating that precursor cells, rather than the BM environment, are the target of the disease process in AA. An acute deficiency of CD34⁺CD38⁻ cells, representing a noncy-

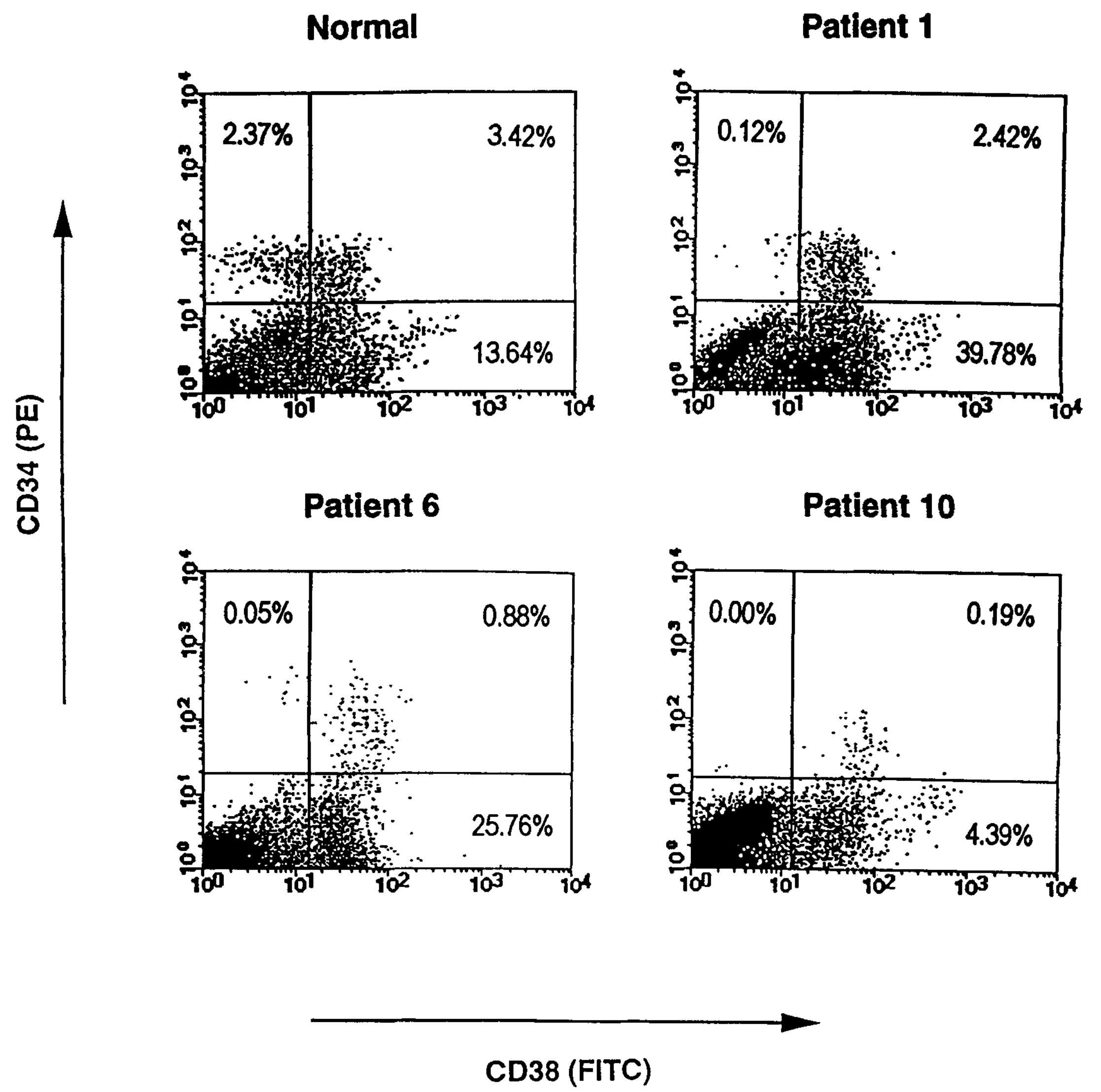

Fig. 6. Flow-cytometric analysis of CD34⁺CD38⁻ and CD34⁺CD38⁺ cells. BM-MNC from normal and AA patient marrow was double-stained with anti-CD34 (PE) and anti-CD38 (FITC) antibodies. Numbers in quadrants refer to percentage of single- and double-stained cell populations.

TABLE IV. Analysis of CD34⁺ Cell Subpopulations of BM Cells in AA*

Cell subset	% of CD34⁺ cells N	% of CD34⁺ cells AA
CD34⁺CD33⁻	58.8 ± 9.9	34.6 ± 7.8
CD34⁻CD33⁺	41.2 ± 9.9	65.4 ± 7.8
CD34⁻CD71⁻	64.7 ± 9.1	58.3 ± 6.8
CD34⁺CD71⁺	35.3 ± 9.1	41.7 ± 6.8
CD34⁺CD45RA⁻	87.4 ± 8.7	75.8 ± 5.7
CD34⁻CD45RA⁺	12.6 ± 8.7	24.2 ± 5.7

*Percentage of lineage-negative and lineage-positive populations of CD34⁺ cells is presented as mean ± SEM of normal (N; n = 5) and AA (patients 1–10) values.

cling population with relatively little colony-forming ability [30–32], was not obvious when growth of isolated CD34⁺ cells was examined in clonogenicity assays. However, since CD34⁺CD38⁻ cells represent a population capable of sustaining hematopoiesis in vitro in long-term cultures [30], their deficiency in AA may provide an explanation for the results of Marsh et al. [13], who demonstrated a poor regenerative capacity of CD34⁺ cells grown on heterologous stroma layers.

In summary, the disease process in AA depletes the pool of the most immature CD34⁺CD38⁻ and CD34⁺*c-kit*⁺ subpopulations of hematopoietic progenitors. A tendency to enhanced differentiation in AA was confirmed by an increase in the proportion of CD34⁺ cells coexpressing the lineage-commitment antigens CD33, CD71, and

CD45RA. It appears that the damage to early precursor cells by the disease process is irreversible, and might be comparable to the irreversible damage to β-islet cells in autoimmune-type diabetes. An excess of cytotoxic activated T cells in BM of patients in the acute phase of the disease and reportedly elevated during hematopoietic recovery [33,34] may be responsible for depletion of the stem-cell compartment in AA by an ongoing autoimmune mechanism. Persistent abnormalities of early hematopoietic progenitors observed in the majority of patients in clinical remission support the recommendations that BMT or immunosuppressive therapy in AA should be performed early [35] before irreversible damage to stem cells has occurred.

Despite low numbers and abnormal proportions between immature and more differentiated progenitors, indicative of a persistent defect in the stem-cell compartment, most AA patients reach hematological remission after immunosuppressive treatment. This suggests the existence of yet-unknown compensatory mechanisms, allowing normal blood-cell counts from low numbers of $CD34^+$ cells and their early subsets to be maintained. Previous work from our laboratory [36] has documented permanent morphological changes in bone marrow and peripheral blood of patients treated with ALG. In vivo normalization of hematological values after ALG is likely to be associated with "stressed" hematopoiesis, which compensates for peripheral needs by excess differentiation over the years. This could in part explain why immunosuppressive treatment for severe AA improves survival and the hematologic status, but leaves the patient at risk for late complications, i.e., relapse and progression to hematological malignancies.

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